The role of signaling pathways on proliferation and self-renewal of cultured bovine primitive germ cells

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Title: The role of signaling pathways on proliferation and self-renewal of bovine primitive germ cells in culture.

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Abstract

Purpose Gonocytes are primitive male germ cells residing in the neonatal testes and are unipotent in nature, but also have a pluripotent stem cell ability in mice under appropriate culture conditions. This study was performed to elucidate molecular mechanisms on self-renewal and survival of bovine gonocytes in culture.

Methods Gonocytes were isolated from neonatal bull calves and were culture in DMEM/F12, supplemented with 15% Knock-out serum replacement (KSR) and growth factor glial cell-derived neurotrophic factor (GDNF). Cells were analyzed at 6 days after culture for cell signaling molecular markers.

Results Colony formation was observed 3-4 days after culture. The addition of GDNF enhanced mitogen-activated protein kinase 1/2 (MAPK1/2) phosphorylation and activated the MAPK signaling pathway. The inhibition of MAPK signaling reduced cell proliferation and abolished colony formation. However, the inhibition of phosphoinositide 3-kinase-AKT (PI3K-AKT) signaling, a dominant pathway for self-renewal of mouse germ cells, did not show any effects on cultured bovine gonocytes. The expression of cell cycle-related regulators cyclin D2 and CDK2 (cyclin-dependent kinase 2) was downregulated with the inhibition of MAPK signaling.

Conclusions These results indicate that the activation of MAPK plays a critical role in the self-renewal and survival of bovine gonocytes via cyclin D1 and CDK2.

Key words: Cell cycle regulators・Gonocytes・ MAPK・Signaling Pathways・Self-renewal・ Testis

Introduction

Gonocytes reside mostly in the center of the seminiferous tubules and remain quiescent [1]. These cells resume proliferation, migrate to the basement membrane and are transformed to spermatogonial stem cells (SSCs) after arriving at a stem cell niche. The niche referred as specialized microenvironments, which provide architectural support, stimulate to secrete growth factors, and provide extrinsic signals to synchronize self-renewal and differentiation [2].

Understanding the niche factor that regulates germ cell function in rodents has been greatly aided by transplantation assays to immunodeficient mice and the development of a long-term culture system [3]. Culture conditions that support the long-term self-renewal and maintenance of pluripotency of germ cells have been established in various species including mice [4, 5, 6] rats [7], hamsters [8], and rabbits [9]. Growth factor GDNF was shown to be the critical factor for the self-renewal of cultured germ cells in these culture systems. Global gene expression profiling has been identified several intrinsic downstream targets for the GDNF-mediated self-renewal of cultured germ cells. Among these targets, Ets variant 5 (Erm), B cell/lymphoma 6 membrane B (Bcl6b), and LIM homeobox1 (Lhx1) have been identified as core transcription factors associated with the self-renewal of cultured mouse germ cells [10].

The combined approach of RNAi inhibition, microarray analysis, and transplantation assays has revealed the cascade of self-renewal and pluripotency in cultured germ cells. The *ETV5-Bcl6b-Lhx1* cascade under the influence of GDNF was shown to be responsible for the self-renewal and maintenance of mouse germ cells [11]. This mechanism differs from those of mouse ES cells and human ES cells, in which self-renewal and pluripotency maintain the *Oct4-Sox2-Nanog* network [12]. However, the extrinsic signaling pathways for self-renewal and pluripotency respond differently in mice and human ES cells. Instead of different growth factor requirements, common signaling pathways play opposite roles in mice and humans; for example, MAPK inactivation is required for self-renewal in mouse ES cells, while it induces differentiation in human ES cells [13]. Studies on extrinsic signaling pathways of germ cell cultures in mice using a kinase–specific inhibitor demonstrated that PI3K-AKT signaling [14, 15, 16] and Ras-mediated MAPK signaling [17, 18] were

involved in the self-renewal and survival of germ cells. Crosstalk between PI3K/AKT and MAPK signaling was also shown to be essential for the self-renewal of cultured mouse germ cells [15].

In domestic species, gene targeting has a potential application in both agriculture and human disease modeling. A combination of gene targeting and pluripotent germ cell lines will provide a time-saving and cost-effective tool for maximizing genetic gain and preserving desirable genetics for the production of superior food animals [19]. The major hindrance in the practical application of this research is the lack of a long-term culture system supporting the self-renewal of germ cells in domestic species. Although germ cells from many mammalian species have been shown to proliferate for more than six months in the seminiferous tubules of immunodeficient mice [20], no germ cell line has been established in livestock species. A possible reason for this is the dearth of understanding on species-specific requirements of growth factors and mechanisms supporting the self-renewal of cultured germ cells.

In the present study, we focused on exploring the molecular mechanisms responsible for the self-renewal and maintenance of cultured bovine primitive germ cells (gonocytes). Our results indicated that activation of the MAPK pathway was necessary for the self-renewal and maintenance of cultured bovine gonocytes via the downstream regulation of cyclin D1 and CDK2.

Materials and methods

Collection of the testes and isolation of gonocytes

The testes were collected from 0 to 10-day-old Holstein or Japanese Black bull calves in Dulbecco's modified Eagle's medium and Ham's 12 (DMEM/F12; GIBCOBRL Invitrogen, Carlsbad, CA, USA) supplemented with 15 mM HEPES (Wako Pure Chemical, Tokyo, Japan) from National Livestock Breeding Centre (Fukushima), Gifu Prefectural Livestock Research Institute (Gifu) and Livestock Farm (Kyoto) and were transported to the laboratory on ice within 24 hours.

Gonocytes were isolated by a three-step enzymatic digestion method as described previously [21] with minor modifications. Briefly, the testes were decapsulated and minced, and the minced tissues were digested with collagenase Type IV (1 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 45 min with constant agitation. After three washes, tissue fragments of the seminiferous

tubules were incubated with collagenase Type IV and hyaluronidase (1 mg/ml; Sigma Aldrich). The cell suspension was further incubated with a mixture of 0.25% trypsin (Nacalai Tesque, Kyoto, Japan) and DNase I (7 mg/ml; Sigma Aldrich) for 10 min. After centrifugation, the resulting pellet was suspended in DMEM/F12 medium containing 10% FBS to stop the enzymatic activity of trypsin. The cell suspension was filtered through a 40 μm nylon mesh (Kyoshin Rikou, Tokyo, Japan) and suspended in DMEM/F12 medium containing 5 % FBS.

The cell suspension was subjected to Percoll density gradient (20%-60%) centrifugation at 3400 g for 30 min at 21 °C. Cells from the fraction between 35 to 45 % Percoll were separated and plated on 0.2% gelatin-coated dishes (Sigma-Aldrich) for 6 hours in DMEM/F12 medium containing 5% FBS. The supernatant containing germ cells was collected and utilized for further experiments.

Cell culture and treatments with cell signaling inhibitors

The culture medium for gonocytes was used DMEM/F12, which was supplemented with 15% Knock-out serum replacement (KSR) (GIBCOBRL, Invitrogen, Carlsbad, CA, USA), 10 μg/ml apotransferrin (Sigma Aldrich), 10 μg/ml insulin (Sigma Aldrich), 110 μg/ml sodium pyruvate (Sigma Aldrich), 0.015% sodium DL-lactate (Sigma Aldrich), NEAA (non-essential amino acid solution, GIBCOBRL Invitrogen, Carlsbad, CA, USA), 100 μM β-mercaptoethanol (Wako Pure Chemical, Tokyo, Japan), 100 μg/ml penicillin (Sigma Aldrich), 50 μg/ml streptomycin (Sigma Aldrich), and 40 μg/ml Gentamycin (Sigma Aldrich) with 1% FBS. GDNF (40 ng/ml, R&D, Minneapolis, MN, USA) was used as a growth factor in this study.

Culture dishes were coated with 0.001% poly-L-lysine (P2658, Sigma Aldrich) for 1 hr at 37 °C. The dishes were washed once with PBS and utilized for cell culture. Isolated cells were plated at a density of 5×10^5 cells per well of a 6-well dish (Becton Dickinson, Franklin Lakes, NJ, USA) pre-coated with poly-L-lysine and cultured at 37ºC for 6 days in 5% CO_2 in air.

Inhibitors of the MEK (PD098059, Stemgent, USA) (PD) and PI3K (LY294002, Cell Signaling, Beverly, MA USA) (LY) signaling pathways were used at a dose of 10 μM [16]. The inhibitor treatment was given on day 3 of culture. Colonies were photographed and counted manually using an inverted microscope (Nikon, DIAPHOT-300, Tokyo, Japan).

Immunofluorescence

Cell smears were prepared on poly-l-lysine-coated glass slides. To stain colonies, cells were cultured for 6 days onto coverslips in 24-well culture dishes (Nunc, DK-4000, Roskilde Denmark). The procedure was performed as described previously (Kim *et al.* 2013). Briefly, cells were fixed in 4% paraformaldehyde for 10 min and incubated with 10% goat serum in TBS-T (Tris buffered saline containing 0.1% Triton X-100) for 1 hr at 37 °C. Samples were washed thrice and incubated with primary antibodies at the optimal concentration overnight at 4 °C. The antibodies are used as anti-VASA (1:300; Chemicon, USA) and anti-PGP9.5 (1:200; Biomol, Exeter, Exeter, UK). Samples were again washed thrice and incubated with secondary antibodies such as anti–mouse or anti–rabbit IgG antibodies conjugated with FITC (1:200; DAKO A/S, Denmark) along with DBA-Rhodamine (1:100; Vector Laboratories, Burlingame, USA). The samples were counterstained with DAPI mounting media (Vector Laboratories, Burlingame, CA USA) for 10 min. For negative control, primary antibodies were omitted and the sections was incubated with secondary antibodies Mouse normal IgG, (1:200 dilution, Santa Cruz Biotchnology) and Rabbit normal IgG, (1:200 dilution, Santa Cruze, USA). Photographs were taken with the inverted fluorescent microscope, Eclipse TE2000-U (Olympus BX50, Tokyo, Japan).

RNA isolation and RT-PCR

Total RNA was isolated with Trizol reagent (Invitrogen) according to the manufacturer's protocol. Complementary DNA was synthesized from 1 µg total RNA using ReverTra Ace (MMLV reverse transcriptase RNaseH; Toyobo, Osaka, Japan). To rule out genomic DNA contamination, reactions were performed for samples without the addition of ReverTra Ace (RT-). PCR amplification was performed using 1µL cDNA per 20 µL PCR reaction mixture containing 2 mM MgCl2, 0.25 mM dNTPs, 1× PCR buffer, 5 pmol of each primer, and 1U Taq DNA polymerase (ExTaq; TaKaRa, Tokyo, Japan). Primer sequences are shown in Table 1. PCR products were separated by 1.5% agarose gel electrophoresis and stained with 0.5 µgmL^{-1} ethidium bromide. All PCR products were sequenced to confirm their identity.

Western blot analysis

Isolated cells including gonocytes were cultured for 3 days and were then treated with GDNF, PD and/or LY for 20 min. These cells were lysed in Radioimmunoprecipitation assay buffer (RIPA)

buffer to obtain protein lysates (Abcam, Cambridge, England). Protein concentrations were determined using Coomassie Bradford reagent (Sigma Aldrich). Fifty µg of total protein was mixed with an equal amount of 2x-SDS loading buffer and resolved by SDSPAGE. Electrophoresis was performed using a Mini electrophoresis system (Biocraft, Tokyo, Japan) at 100V for 60 min. The eluted proteins were transferred to an Immobilon-P transfer membrane (Millipore, Massachusetts, USA) at 60V for 90 min. The transmembrane was blocked for nonspecific antibodies with 5% BSA in TBS-T for 90 min at room temperature with gentle shaking. Blots were probed with the primary antibody anti-rabbit pERK (1:5000; Santa Cruz Biotechnology, USA), anti-rabbit p44/42MAPK (1:5000; Cell Signaling, Beverly, MA USA), or anti-mouse α-tubulin (Sigma Aldrich) overnight at 4 °C with gentle shaking. After a brief wash of membranes with TBS-T, membranes were incubated in the secondary antibody ECL-peroxidase labelled anti-rabbit or anti-mouse antibody (1:50000, GE Healthcare, Wisconsin, USA) for 90 min with gentle shaking at room temperature, were washed thrice with TBS-T, and were then developed with an Amersham ECL prime western blotting detection reagent on x-ray film (GE Healthcare, Wisconsin, USA). Density measurements were taken using Imaj J software on scanned x-ray films and normalized using control antibody anti-mouse α-tubulin.

Statistical analysis

All quantification data were presented as the mean ± s.e.m. Analysis of variance (ANOVA) and Turkey's multiple comparison tests were performed using Graph Pad Prism 4.0 (Graph Pad Software, Inc., San Diego CA, USA). Differences were considered to be significant at $P < 0.01$. A densitometric evaluation of western blotting was conducted using Imaj J software with α-tubulin as an internal control.

Results

Gonocytes enrichment and characterization

The enriched gonocytes using Percoll density gradient and differential plating using gelatin-coated dishes were characterized using germ-cell markers DDX4 (Fig. 1A) and PGP9.5 (Fig. 1B).

Effect of the MAPK signaling pathway on self-renewal of cultured germ cells

To investigate the signaling pathways responsible for the self-renewal of gonocytes,

pharmacological inhibitors of the MAPK (PD) and PI3K (LY) signaling pathways were used. Culturing cells in the presence of PD significantly reduced the proliferation of gonocytes and failed to form colonies. However, proliferation and colony formation were not influenced by the presence of LY in the culture (Fig. 2A and B). The appearance of colonies was suppressed in the presence of PD (Fig. 2C).

Western blot analysis indicated that the level of MAPK phosphorylation induced in the culture was higher in the presence of GDNF than in the absence of GDNF (Fig. 2D). MAPK phosphorylation was blocked by the addition of PD to the culture medium, but was unaffected by the addition of LY (Fig. 2D).

Enhanced cell cycle regulation of cultured germ cells

The expression patterns of cell cycle regulators in cultured cells treated with signaling inhibitors were analyzed using RT–PCR (Fig. 3A). The addition of GDNF enhanced the expression of cyclin D2 and CDK2 (Fig. 3B, C, and F, respectively). The expression of cyclin D1 and CDK2 was significantly reduced by the addition of PD to the culture medium (Fig. 3B and F). However, the enhanced expression of cyclin D2 was significantly reduced by the PD treatment (Fig. 3B). The expression of cyclin D3 was unaffected by the addition of GDNF or PD to the culture medium (Fig. 3D and E). Treatment with the LY inhibitor (PI3K signaling) did not influence the expression of these genes.

Discussion

Signaling pathways that regulate the self-renewal and differentiation of germ cells in culture have been well documented in mice [22]. However, the mechanisms of proliferation of cultured germ cells have yet been elucidated in species other than mice. Although several attempts have been made to develop a long-term culture system for bovine gonocytes, colony formation could not obtain after subsequent passages [23, 24]. We previously established a long-term culture system of bovine gonocytes for more than 1.5 months [25]. In this study, culture condition was stable to maintain cell survival and proliferation of bovine gonocytes, mouse embryonic stem (ES)-like colonies appeared in culture and expressed pluripotent marker genes (*OCT3/4* and *NANOG*) [25].

GDNF was shown to be a molecule that regulates self-renewal and differentiation of mouse

SSCs [26]. GDNF signals act though the multicomponent receptor complex comprised of GFRα-1 and RET tyrosin kinases in various cell types [27]. GFRα-1 and RET have also been recognized as spermatogonial markers expressed in gonocytes, SSCs, and differentiated spermatogonia [28]. These co-receptors of GDNF-mediated signaling were shown to be necessary for the self-renewal of germ cells in rodents [29]. The GDNF enhanced cell proliferation and colony formation bovine gonocytes were reported by Aponte et al [30, 31], which indicated that GDNF-mediated signaling was conserved in germ cell cultures in rodents and cattle.

In the present study, we showed that the inhibition of MAPK pathways by the inhibitor PD98095 impaired cell proliferation and abolished colony formation (Fig. 2A and 2B). The presence of GDNF significantly increased tyrosine phosphorylation of MAPK44/42 (Fig. 3D and 3E). This stimulation was blocked by the treatment with PD98059 (Fig. 3D and 3E). These results indicate that the activation of MAPK pathways is essential for the self-renewal of bovine gonocytes in culture. In accordance with these results, GDNF signals were previously shown to activate RET phosphorylation and subsequently activate MAPK pathways, which are essential for the cell growth and proliferation of SSCs in mice [17]. Previous studies also demonstrated that FGF2, not GDNF, mediates the activation of the MAPK pathway by upregulating the downstream targets ETV5 and Bcl6b in mouse germ cell culture [32]. However, the addition of FGF2 to our culture system enhanced somatic cell proliferation and induced the differentiation of gonocytes (unpublished data).

PI3K/AKT is known to play an important role in the self-renewal of germ cells in mice through GDNF or FGF2 stimulation [33]. The activation of PI3K/AKT signaling in mouse germ cells was shown to be completely inhibited by the inhibitor LY294002, which impaired the self-renewal of cultured germ cells [15, 16]. However, the activation of AKT alone was not sufficient for the self-renewal of SSCs [15]. Src kinase is an alternative activator of PI3K pathways, which results in the upregulation of *N-myc* expression and promotes the proliferation and self-renewal of mouse germ cells [14, 16]. Our results showed that the inhibition of PI3K/AKT signaling by LY294002 did not affect the cell proliferation or colony formation of bovine germ cells. This result indicated that AKT- or Src-mediated PI3K signaling did not play a significant role in the self-renewal of bovine gonocytes in culture. This finding is in contrast to that reported in mice, in which PI3K was shown to be the

dominant signaling pathway.

The inhibition of MAPK and PI3K signaling was previously shown to result in the downregulation of pluripotency genes *OCT3/4*, *NANOG*, and *SOX2* in human ES cell lines [34, 35], which indicated that these signaling pathways play essential roles in maintaining the self-renewal and pluripotency of human ES cells. PI3K/AKT signaling was also shown to regulate expression of the self-renewal cascade genes *Bcl6b, Etv5, and Lhx1* germ cell culture in mice [16]. Interestingly, the expression of *Oct3/4* was essential for the survival of mouse germ cells, but was not influenced by GDNF and did not play a significant role in self-renewal [36]. However, the expression of *OCT3/4* and *NANOG* was detected in bovine gonocytes in culture [25] and gonocytes in the testes of pigs [37] and cattle [25], suggesting that these pluripotent genes have roles in the maintenance and self-renewal of gonocytes in domestic species. In contrast, *Nanog* expression has not been detected in cultured bovine gonocytes or in the testes of mice [38]. Our previous report [25] demonstrated that the strong expression of the pluripotency markers *OCT3/4* and *NANOG* in cultured bovine germ cells was associated with the appearance of mouse ES-like colonies. These results indicate that the different expressions of transcription factors in mice and domestic species may lead to different regulatory mechanisms for the self-renewal and colony formation of cultured germ cells. However, the role of these genes has to be elucidated further to understand the MAPK-mediated self-renewal of bovine gonocytes germ cells.

Activation of the extrinsic MAPK [17, 18] and PI3K [15] signaling pathways in mouse germ cells was previously shown to be involved in the regulation of cell-cycle-related cyclin gene expressions. To understand the relationship between signaling pathways and the self-renewal of bovine gonocytes in culture, we analyzed the downstream genes potentially involved in cell cycle regulation. Cyclin D1 is essential for the entry to the G1/S-phase of the cell cycle in the presence or absence of GDNF and is regulated by the MAPK pathway [39]. The expression of cyclin D1 has also been observed in proliferating germ cells and SSCs in the mouse testes [40]. In this study, the expression of cyclin D1 was significantly downregulated after the inhibition of MAPK signaling by PD (Fig. 3A and 3B), but was unaffected by the presence of GDNF. In contrast, cyclin D2 expression

was significantly upregulated upon GDNF stimulation and inhibited upon pre-treatment with the MAPK inhibitor (Fig. 3A and 3C), which indicated that the MAPK pathway was involved in regulating the cell cycle of bovine gonocytes. The overexpression of cyclin D2 was previously shown to regulate the self-renewal of germ cells and was mediated by Ras activation in mice [18]. CDK2 has been shown to be involved in controlling the entry to the S-phase in association with cyclin A. CDK2 was upregulated in the presence of GDNF and controlled entry to the G1/S-phase of mouse C18-4 germ cell lines via MAPK-mediated signaling [17]. In this study, CDK2 expression was also significantly upregulated upon GDNF stimulation and the inhibition of MAPK signaling resulted in the downregulation of CDK2 expression (Fig. 3A and 3E). Enhanced CDK kinase activity was previously shown to be essential for the Ras-induced proliferation of cultured mouse germ cells [18]. Our results suggested that cell cycle-related genes were not influenced by the inhibition of PI3K signaling. This is consistent with a previous report [16], in which the inhibition of PI3K signaling does not significantly affect changes in cyclin gene expression in mouse germ cells in culture.

Taken together, these findings reveal the unique and crucial role of MAPK signaling in maintaining the self-renewal and colony formation of bovine gonocytes in culture. In contrast to our findings, cultured mouse germ cells require the crosstalk between MAPK and PI3K signaling pathways for self-renewal. The downstream targets of MAPK signaling that ultimately influence the self-renewal of bovine gonocytes need to be determined in future experiments. The present study has revealed the marked differences in the control of the self-renewal and survival of cultured germ cells in mice and cattle. These results will be useful for identifying optimal culture conditions to establish a long-term culture system and germ-cell lines in domestic species.

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Disclosures

Conflict of interest: Mahesh Sahare, Ayagi Otomo, Kana Komatsu, Naojiro Minami, Masayasu Yamada, and Hiroshi Imai declare that they have no conflict of interest.

Animal studies: All institutional and national guidelines for the care and use of animals were followed.

Human rights: This article does not contain any studies with human subjects performed by any of the authors.

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411

Figure Legends

Figure 1. Immunocytochemical characterization of cultured gonocytes in the presence of GDNF by using germ cell-specific antibodies (DDX4 and PGP9.5). A) DDX4 expression with the nuclear marker DAPI, B) PGP9.5 expression with DAPI, E) anti-rabbit IgG as a control (Magnification= 40X).

Figure 2. Effects of MAPK and PI3K signaling inhibitors on the self-renewal and colony formation of cultured gonocytes. A) Cell proliferation of cultured gonocytes for 6 days in the presence of MAPK (PD) and PI3K (LY) inhibitors. Cell proliferation was significantly inhibited in the presence of PD relative to that in the absence of GDNF as a control (GD-), in the presence of GDNF (GD+) or LY. **P*< 0.01 and ***P*< 0.01 significantly different from GD-, respectively; $^{\#\#\#\#}P$< 0.01 significantly different from GD+. (Data were collected n=3 in each experiment, from three independent experiments, and indicated as the mean ± s.e.m). B) Colony formation by cultured gonocytes for 6 days in the presence of MAPK/PI3K inhibitors. The number of colonies formed was significantly less in the PD-treated group than in the GD- group. The number of colonies formed was higher in the GD+ culture than in the GD- culture. *****P*< 0.01, ****P*< 0.01 and ***P*< 0.01 significantly different from GD-; $^{\#\#\#\#}P$< 0.01 significantly different from GD+. (Data were collected n=3 in each experiment, from three independent experiments, and indicated as the mean ± s.e.m). C) Appearance of colonies in the control (GD-), and in the presence of GD+, PD, and LY (Magnification= 100X). D) Western blot analysis of MAPK and phosphorylated MAPK (pMAPK). (Gonocytes were cultured for 4 days in the presence of GDNF. As a control, cells were starved for 16 hours without GDNF and treated with no chemicals, PD and LY for 20 min. E) Estimation of phosphorylated MAPK expression based on western blot from three independent immunoblot experiments (mean ± s. e. m). The level of phosphorylated MAPK was significantly lower in PD-treated cells than in GD+ treated cells (***P*< 0.01). However, the level of phosphorylated MAPK was not significantly different in the absence of GD (GD-) and in the presence of LY.

Figure 3. Effect of the inhibition of MAPK and PI3K signaling on the expression of cell cycle regulator genes. A) RT-PCR analysis of cell cycle regulator genes (cyclin D1, cyclin D2, cyclin D3 and CDK2) and b-Actin as the housekeeping gene. Relative mRNA expression of cyclin D1 (B), cyclin D2 (C), cyclin D3 (D), and CDK2 (E) were examined in the presence of GDF or MAPK/PI3K inhibitors. Data represented from three independent gel images (mean ± s.e.m). *P< 0.01 and **P< 0.01 significantly different from GD-, $^{\#}P$< 0.01 and $^{\#\#}P$< 0.01 significantly different from GD+.

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Figure 1.

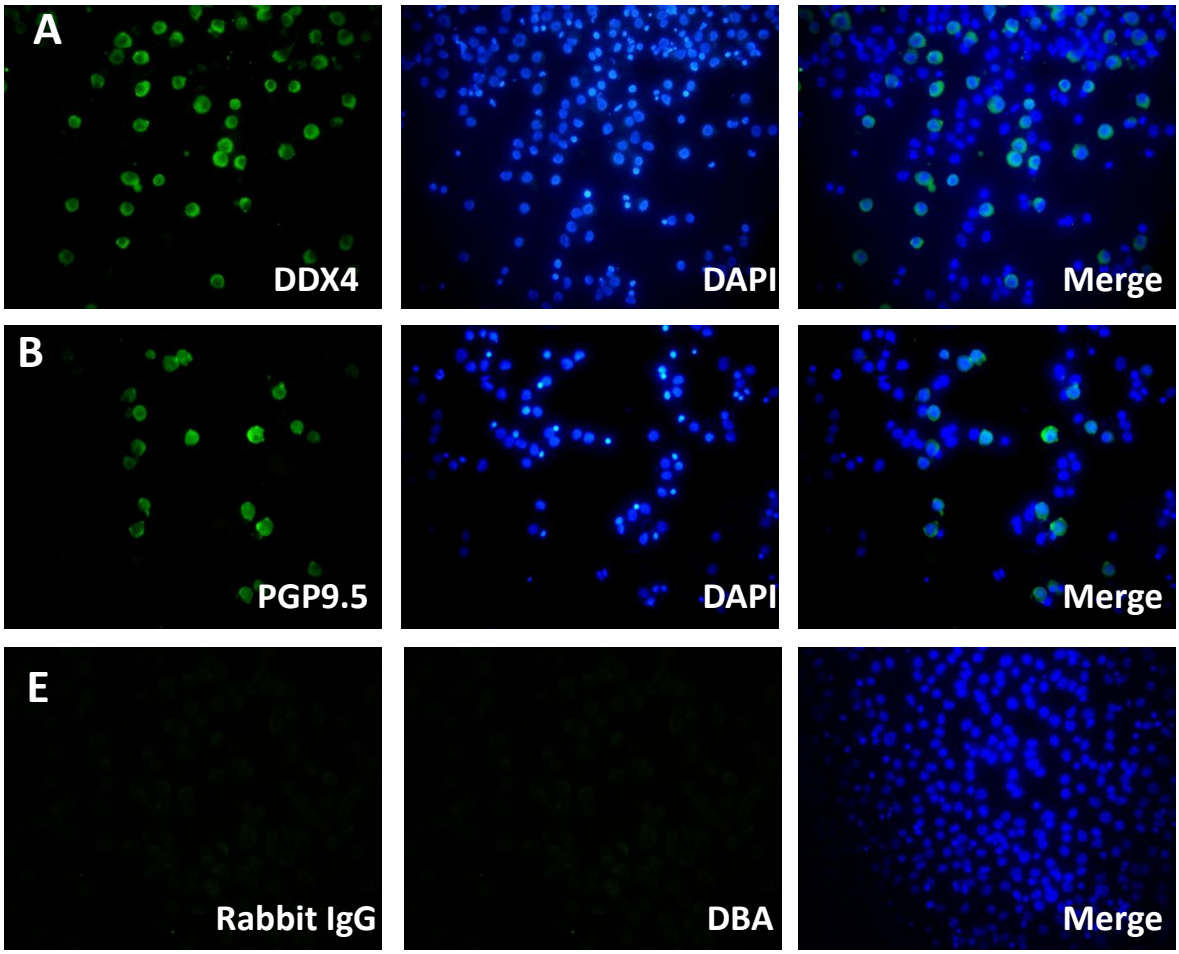
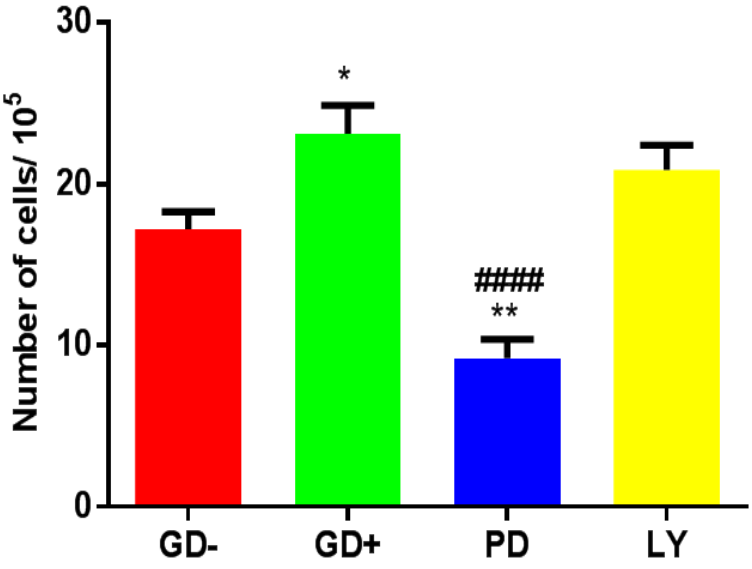


Figure 2.

A



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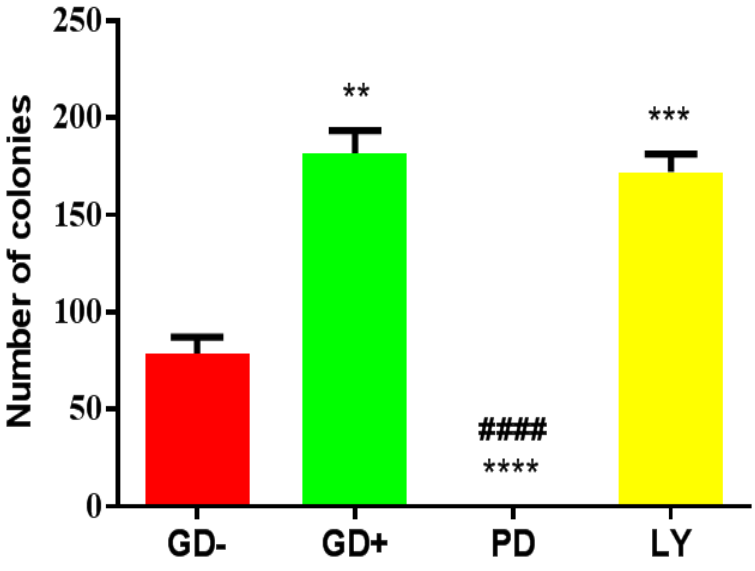
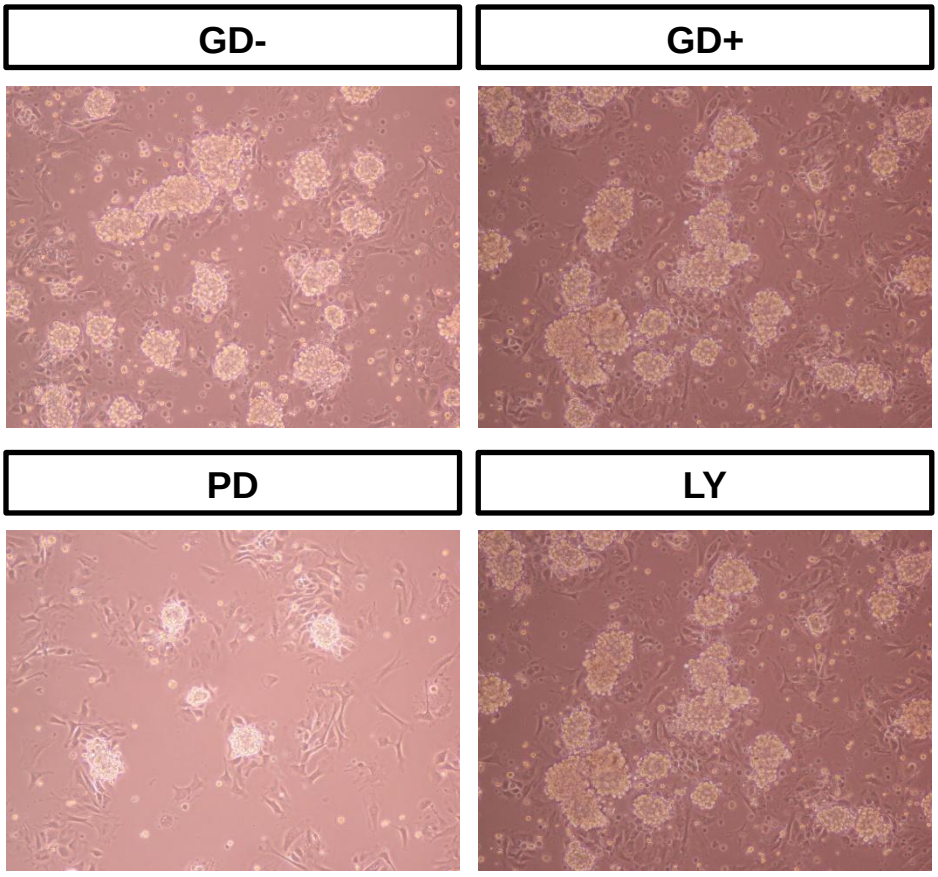


Figure 2.

C



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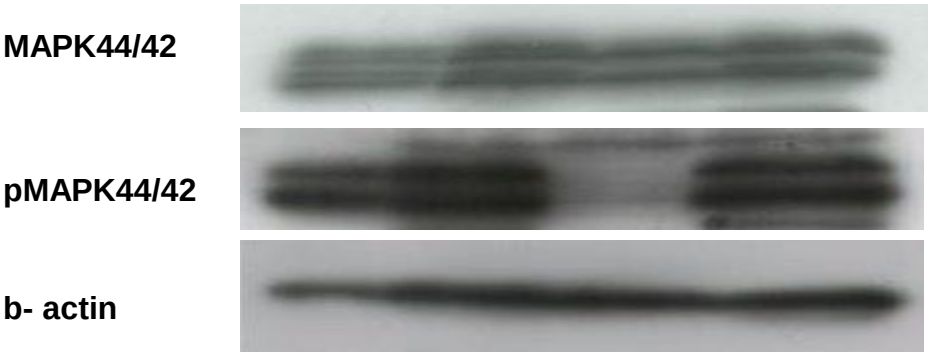


GD	-	+	+	+
PD	-	-	+	-
LY	-	-	-	+

E

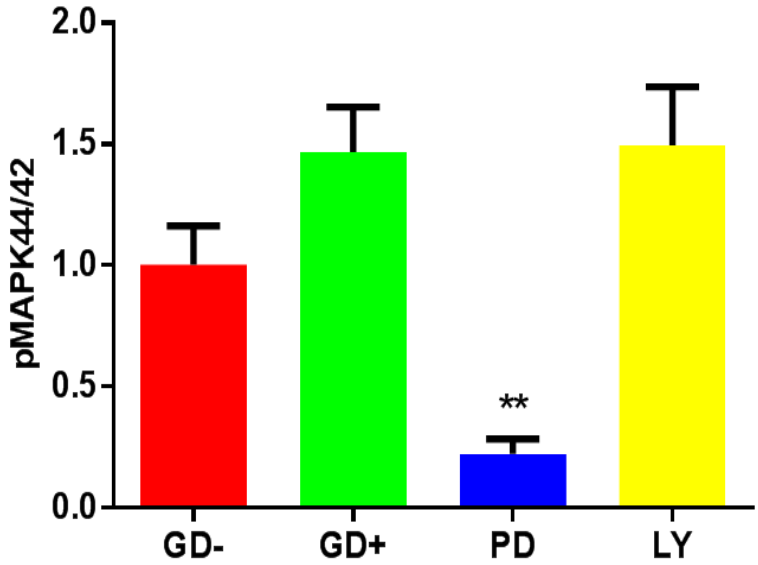
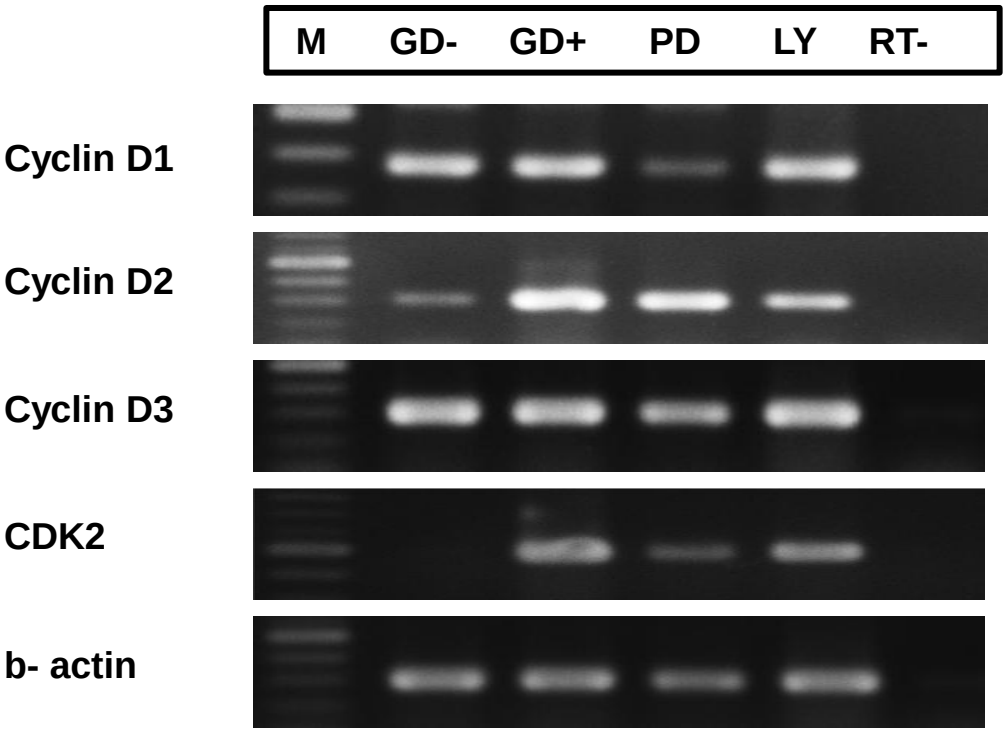


Figure 3.

A



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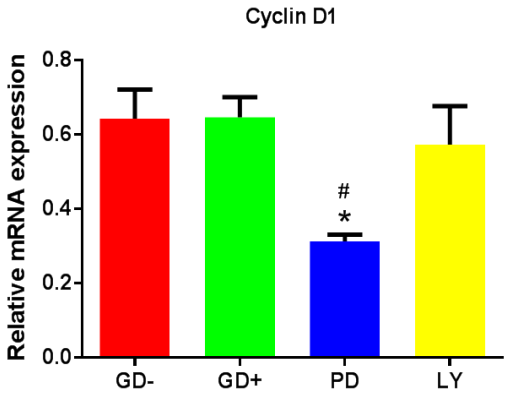

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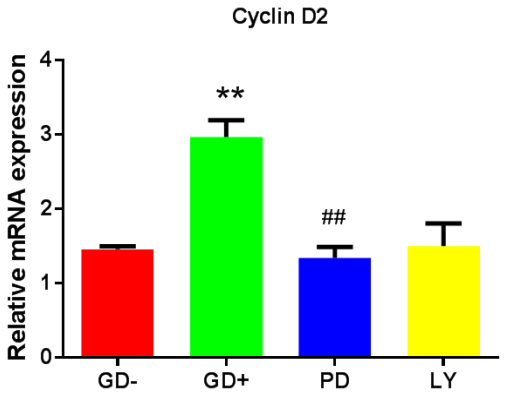

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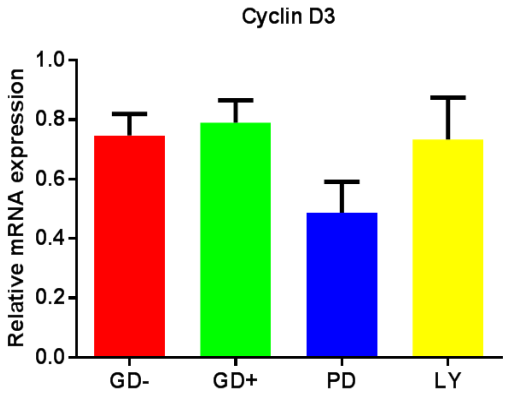

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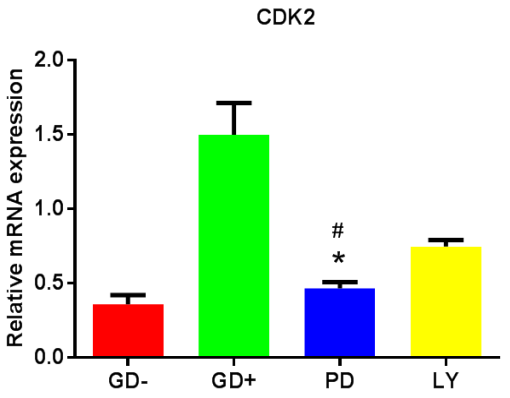

Table 1. RT-PCR primer sequences used for the amplification of specific genes

Gene Name	Primer sequence (5'-3')	Product Size (bp)	Accession no.
Cyclin D1	F: GCCGAGGAGAACAAGCAGAT R:TCAGATGTTCACGTCACGCA	378	NM_001046273.2
Cyclin D2	F: GCAGAACTTGCTGACCATCG R: AGGCTTGATGGAGTTGTCGG	319	NM_001034709.2
Cyclin D3	F: CACTTGGAGGCCCTGCATAA R: GGTAGCATGATGGTCCTCGG	495	NM_001034709.2
CDK2	F: GGGAACGTACGGAGTTGTGT R: CCAGAAGGATTTCCGGTGCT	491	NM_001014934.1
b-Actin	F: CGATCCACACAGAGTACTTGCG R: CGAGCGTGGCTACAGTTCACC	316	NM_173979.3